The neuronal clusters of the accessory bulbar white matter in rabbits. A novel brain structure

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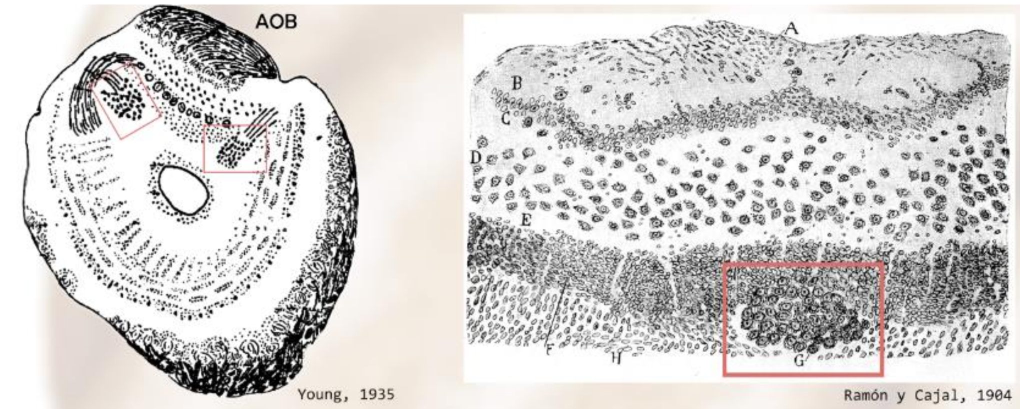
Introduction

The accessory olfactory bulb (AOB) is the first neural integrative centre of the vomeronasal system (VNS). There are significant species-specific differences in the structure of the AOB between mammals. Our previous structural and immunohistochemical study of the rabbit AOB (Villamayor *et al.,* 2019) has demonstrated its full development in terms of lamination and functionality. The examination of the neuronal organisation of the rabbit AOB was completed with the microscopical study of the neuronal clusters encased within the accessory bulbar white matter, immediately beneath the AOB granular cell layer. Following their first description by Ramón y Cajal (1904) in guinea pigs (Fig.1B), these sub-bulbar structures have received very little attention. It was just Larriva-Sahd (2012) who has studied these structures comprehensively in rats, mice, and guinea pigs, linking them with the transitional zone of the AOB. Our findings reveal that these sub-bulbar structures are more complexly organised in rabbits than has previously been described for other mammalian AOBs. With this study, we have addressed the existing knowledge gap regarding the structural and immunohistochemical characterisation of these clusters. We have employed microdissection, specific histological staining techniques and some immunohistochemical markers.



Young, 1935

Fig. 3 Transverse section through the accessory olfactory and the rostral extreme of the nucleus olfactorius anterior of the rabbit. Toluidin blue preparation.

Ramón y Cajal, 1904

Fig. 745.—Trozo del robusto lóbulo accesorio del conejo de Indias. (Método de Nissl). A, zona fibrilar; B, capa glomerular; C, zona de los granos superficiales; D, capa de las células empenachadas; E, substancia blanca; F, granos; H, células epiteliales; G, foco de neuronas gruesas.

Fig 1. Representation of the neuronal clusters of the AOB. (A) Transversal section of the rabbit AOB showing two of these clusters (Young, 1935). (B) Sagittal section of the guinea pig AOB showing a neuronal cluster beneath the granular cell layer (Ramón y Cajal, 1904).

Material & Methods

Ten adult healthy rabbits of both sexes aged from at least 3.0 months to 1 year old were provided by an abattoir. All of them were commercial Hyplus hybrid (Grimaud Frères, France). The rabbits were humanely sacrificed under current legislation [Council Regulation (EC) 1099/2009], and the heads were separated from the carcasses in the slaughtering line. All procedures followed the guidelines for housing and handling provided by the Bioethical Committee of the University of Santiago de Compostela, and conformed to European legislation (EU directive 2010/63/EU) and Spanish legislation (RD 53/2013). Tal vez esta frase la quitaría pues esa parte hace referencia a los ratones control que aquí no usamos.

After fixation, the olfactory bulbs (OBs) were completely dissected out to be processed. Paraffin wax embedding was the most commonly performed type of inclusion. The OBs were cut into sagittal and transverse serial 5-8 µm sections for the examination of the AOB topography as well as the neuronal clusters. The sections were stained with Nissl and Bielschowsky stainings. Immunohistochemical stainings were performed in paraffin embedded sections.

Bielschowsky's silver stain protocol

Dewaxed and hydrated slides were stained in 20% silver nitrate in dark at 37 °C for 30 min. After 2 x 5 min washes in distilled water, concentrated ammonia was added to the silver solution drop by drop until the precipitate formed was completely dissolved. Samples were then incubated in this solution for 15 min at 37 °C in dark followed by 2 x 10 min 0.1 % ammonia washes. The developer solution is made up of 20 ml formaldehyde 10%, 100 ml distilled water, 0.5 g citric acid, and 2 drops of nitric acid. Such solution was added to the initial silver solution and then the samples were rinsed in it for 10 min. The samples were washed again with ammonia and placed for 1 min in 5% sodium thiosulfate ($Na_2S_2O_3$). In some cases, the sections were counterstained with pyronin. Finally, the slides were washed in distilled water, dehydrated, cleared and mounted.

Immunohistochemical protocol for paraffin-embedded tissue

All primary antibody incubations were performed at 4 °C temperature while the secondary antibodies were incubated at room temperature. Both were kept in a humid chamber during the entire procedures. Unless otherwise stated, all washing steps consisted of three successive 5 min rinses in PB.

The sections were quenched in 3% H_2O_2 for 15 min. Next, non-specific binding was blocked for 30 minutes with 2.5% horse normal serum of the Impress reagent kit Anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA). The sections were then overnight incubated with the primary antibody. The next day the samples were incubated for 20 minutes with the corresponding ImmPRESS VR Polymer HRP Anti-Rabbit IgG Reagent. After rinsing in 0.2 M Tris–HCl buffer (pH 7.6) for 10 min, the sections were finally developed using DAB as chromogen and then dehydrated and mounted.

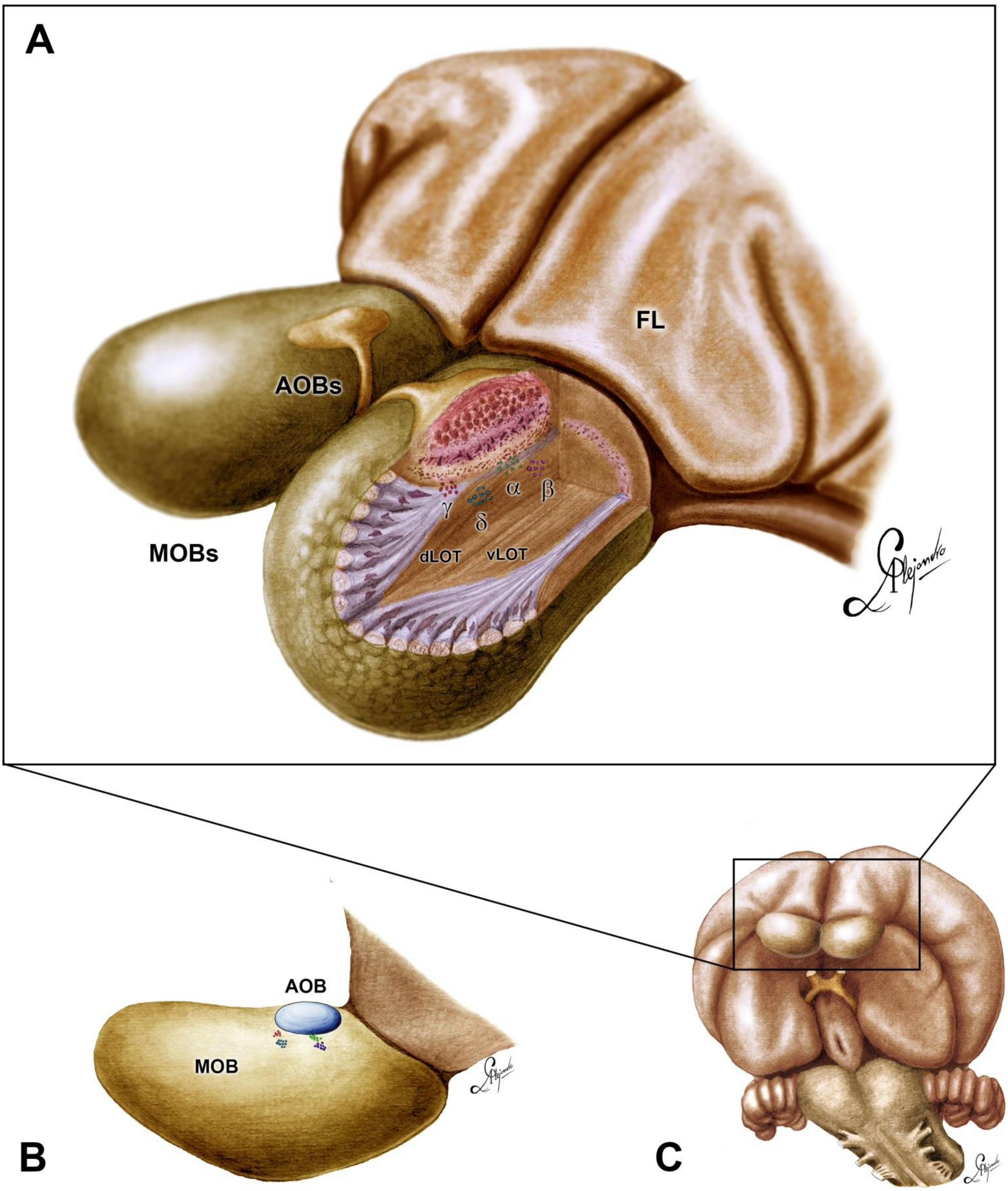
Results

Neuronal clusters in the accessory bulbar core white matter

Nissl-stained serial sagittal sections of the bulb showed four distinct cellular clusters that contained pyramidal-like neurons parallel to the base of the AOB. These nuclei were encased in the accessory bulbar white matter of the lateral olfactory tract (LOT), immediately beneath the granular layer (GrL). They followed an ordered topographical pattern that is demonstrated in Fig. 2. Two of these nuclei were located in the caudal half of the AOB, with one located medially and one located laterally. The other two nuclei were located at the level of the anterior third of the AOB, in a similar fashion, with one located medially and the other located laterally. The caudal nuclei correspond to what have recently been termed the α and β groups of the anterior olfactory nucleus (AON). The α group is situated in the medial aspect of the bulb, whereas the β cluster is located closer to the lateral surface. The two rostral nuclei are described here for the first time. One is located medially, while the other is located laterally. According to the criteria used to name the caudal nuclei, we have named these nuclei the γ and δ groups, respectively.

In all cases, the Nissl sagittal stainings (Fig. 3) showed that the nuclei contain large, deeply stained, oval and polygonal cells. The presence of the four nuclei was also confirmed in our Nissl transverse serial sections (Fig. 5). Pyronin and Bielschowsky stains confirmed that the clusters are encapsulated by the thick fibres of the dorsal LOT (Fig. 4). The immunohistochemical study showed that the nuclei are composed of glutamate-immunoreactive cells (Fig. 4C).

Fig 2. (next page) Drawings of the olfactory bulbs, showing the topographical organisation of the four neuronal clusters identified in the accessory bulbar core white matter. (A) The α and β groups are located parallel to the caudal half of the bulb, whereas the γ and δ groups are beneath the rostral third of the bulb. (B) Schematic drawing of the topographic relationships between the nuclei and the AOB: α (green), β (dark blue), γ (red), and δ (light blue). (C) Rostro-ventral view of the rabbit brain, showing the area depicted in (A). AOB, accessory olfactory bulb; MOB, main olfactory bulb; FL, frontal lobe; dLOT, dorsal lateral olfactory tract; vLOT, ventral lateral olfactory tract.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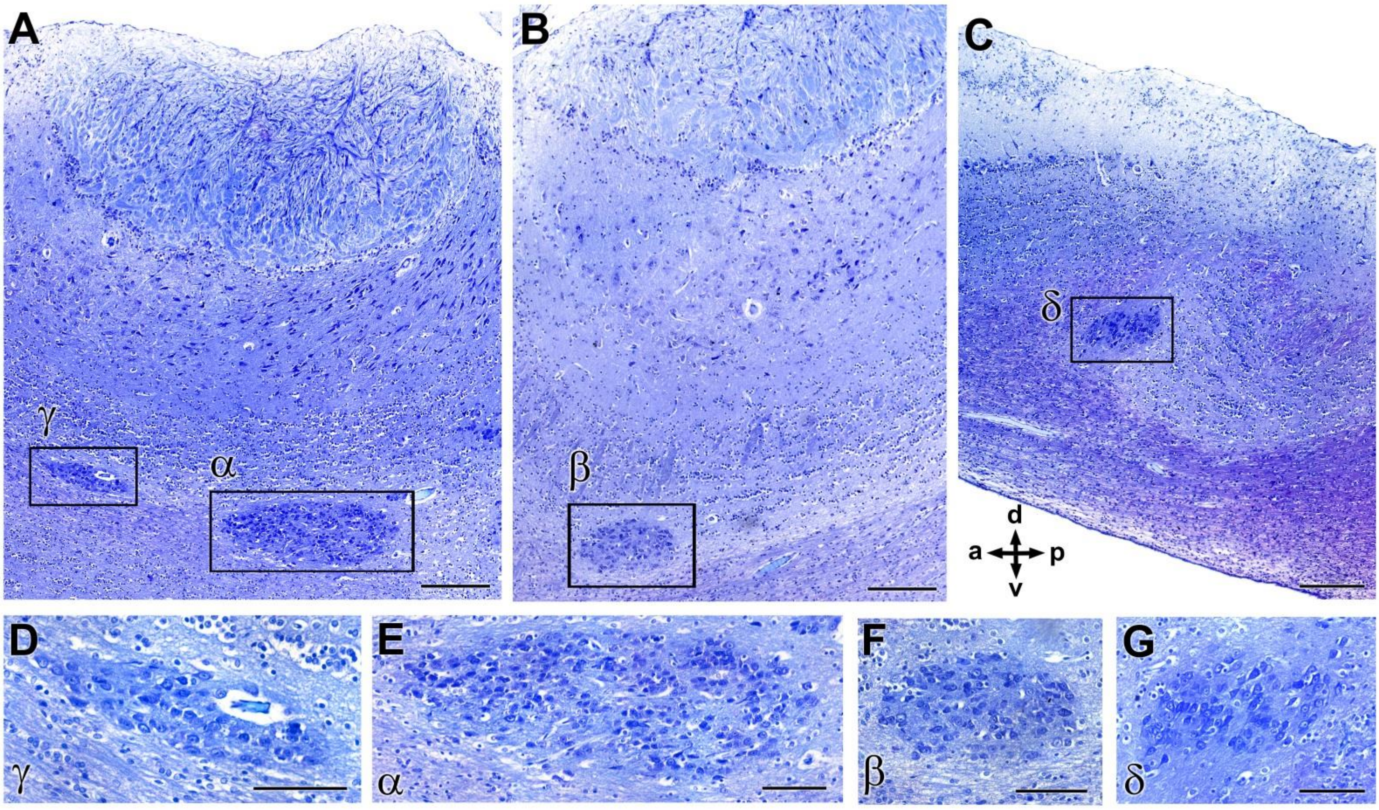

Fig 3. Three selected photomicrographs of the Nissl-stained sagittal serial sections of the AOB, showing the four neuronal clusters present in the accessory bulbar core white matter. The sections are ordered from medial (A) to lateral (C). (D-G) Higher magnification of the four nuclei shown in A-C: γ (D), α (E), β (F), and δ (G). a, anterior; p, posterior; d, dorsal; v, ventral. Scale bars: 250 µm (A-C); 100 µm (D-G).

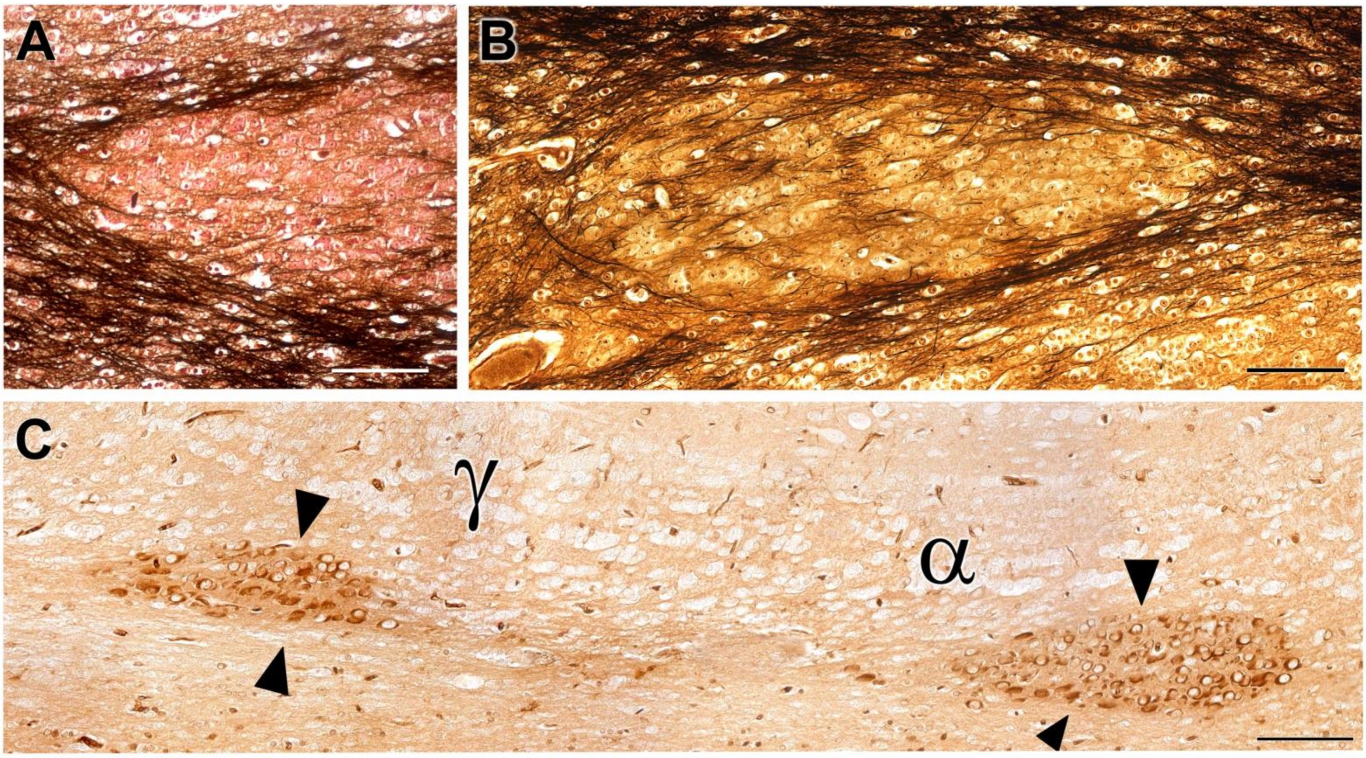

Fig 4. (previous page) Histological and immunohistochemical study of the intrabulbar neuronal clusters. (A) Bielschovsky stained, pyronin contrastained, sagittal section of the α group. (B) Bielschovsky stained section of the α group. (E) Anti-glutaminase immuohistochemical labelling of the α and γ groups. a, anterior; p, posterior; d, dorsal; v, ventral. Scale bars: 50 μm (A); 100 μm (B); 250 μm (C).

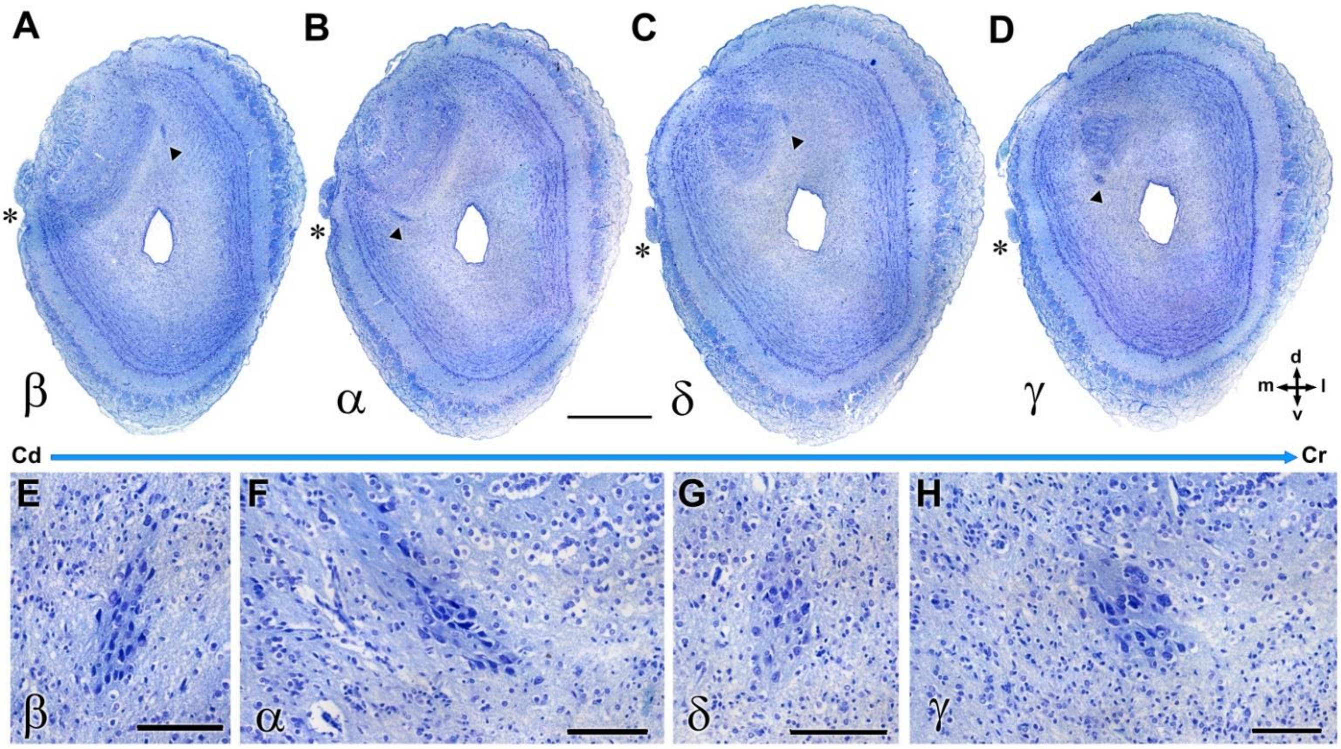

Fig. 5 Four selected photomicrographs of Nissl-stained transverse serial sections of the AOB, containing the four neuronal clusters of the accessory bulbar core white matter. The sections are ordered from caudal (A) to rostral (D). E-H Higher magnifications of the four nuclei shown in A-D: β (E), α (F), δ (G), and γ (H). m, medial; l, lateral; d, dorsal; v, ventral; *, vomeronasal nerve. Scale bars: 1 mm (A-D); 50 µm (E-H).

Discussion

The deepest layer of the rabbit AOB has a striking feature that has received very little attention: the presence, parallel to its ventral segment, of a series of pyramidal-like neuronal clusters that are neatly delimited by the myelinic fibres of the dorsal LOT. One such cluster was first described by Ramón y Cajal in the guinea pig (1904) (Fig 1B), although he was not able to assign it any function. Lohman (1963) studied these clusters in the guinea pig using Nissl staining, assuming that they belonged to the pars rostralis of the AON. Valverde *et al.* (1989), in their study on the hedgehog AON, described the presence of two similar small cell populations in the bulbar portion of the AON. These populations were respectively located in the medial and lateral sections of the dorsal extension of the pars externa of the AON and they were referred to as the α and β groups of the AON. Later, Larriva-Sahd (2012) studied the α-group in rats, mice, and guinea pigs, and reported that the dendrites of these pyramidal-like cells organise into a bundle that ascends to the trigone bounded by the edges of the olfactory limb, the AOB and the dorsal section of the AON. Accordingly, these islands are likely to be functionally related to the sensory qualities detected by both the MOB and AOB.

The only description of this type of cluster in rabbits was reported by Moses Wharton Young (1936) (Fig. 1A), who in his study of the non-cortical centres of the telencephalon of the rabbit, included both groups in a schematic transverse drawing of the caudal part of the olfactory bulb. They were described in the text as circular patches of cells in the medial and lateral extremes of the pars dorsalis of the AON. Undoubtedly, these patches are the rabbit equivalents of the α and β groups of the AON that were described by Valverde *et al. (*1989).

Our sagittal and transverse serial sections of several specimens of the rabbit AOB demonstrate that, in addition to the α and β clusters, the rabbit possesses two clusters topographically related to the anterior AOB, one of which is located medially, whereas the other is found laterally. Following the Valverde criteria, we have named them the γ and δ groups. The anterior positioning of these additional groups makes it very unlikely that they are associated with the AON, supporting the observations of Larriva-Sahd (2012), who associated the α group with the second order processing of olfactory and vomeronasal information. Future studies should address the roles played by these unique neuronal formations.

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